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For: ASSAY METHOD AND KIT THEREFOR

LETTER

Assistant Commissioner for Patents
Washington, DC 20231

October 23, 2001

Sir:

Under the provisions of 35 U.S.C. § 119 and 37 C.F.R. § 1.55(a), the applicant(s) hereby claim(s) the right of priority based on the following application(s):

<u>Country</u>	<u>Application No.</u>	<u>Filed</u>
Sweden	0003662-4	October 11, 2000

A certified copy of the above-noted application is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fee required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By Gerald M. Murphy, Jr. (#28,977)
Gerald M. Murphy, Jr., #28,977

GMM/jeb
1614-0254P

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

Attachment

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PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

IN MENDEL-HARTVIG et al.
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Birch Stewart Kurasch & Bred
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Intyg Certificat

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(71) Sökande *Pharmacia Diagnostics AB, Uppsala SE*
Applicant (s)

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Hjördis Segerlund
Hjördis Segerlund

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ASSAY METHOD AND KIT THEREFOR

Field of the invention

The present invention relates to a method of quantitatively or semi-
5 quantitatively determining an analyte in a sample, especially a high concentration
analyte.

Background of the invention

For qualitative and quantitative determination of an analyte in a sample, a so-
10 called sandwich assay is often used, wherein two receptors directed against different
epitopes of the analyte are incubated with a sample containing the analyte, one of the
receptors being detectable, e.g. through a label conjugated thereto. In a heterogeneous
assay format, the second receptor is immobilized (e.g. coupled) to a solid phase or
provided with a binder component, such as biotin, capable of binding to the solid phase,
15 such as an avidin- or streptavidin-coated solid phase.

Especially in case the analyte is present in the sample in a high concentration, it
is customary to dilute the sample before performing the assay to avoid the use of large
and often costly amounts of immobilized receptor and labelled receptor, respectively, or
to avoid technical difficulties where large amounts of receptors cannot be used. Such
20 dilution is not only laborious but also introduces an additional source of error into the
assay.

There is therefore a need of an assay procedure that avoids the necessity of
dilution.

Summary of the invention

25 It is an object of the present invention to provide a method of performing a
heterogeneous sandwich assay which permits the determination of even a high
concentration analyte in a sample without the need to dilute the sample.

It is another object of the invention to provide a method of performing a
30 heterogeneous sandwich assay which reduces to amounts of capturing and detection
reagents used.

It is still another object of the invention to provide test kits for carrying out the
method.

In one aspect of the present invention there is therefore provided a method of determining an analyte in a sample, especially a high concentration analyte, comprising the steps of:

- a) contacting the sample with a predetermined amount of a receptor which binds specifically to the analyte to form an analyte/receptor complex, said predetermined amount of receptor being in excess of that required to bind all analyte in the sample,
- b) isolating on a solid phase a predetermined fraction of the amount of receptor contacted with the analyte, including analyte/receptor complex and unreacted receptor,
- c) detecting the amount of analyte/receptor complex in said isolated predetermined fraction, and
- d) from the detected amount analyte/receptor complex, determining the concentration of analyte in the sample.

In another aspect of the present invention there is provided a test kit for determining an analyte in a sample, comprising (i) a predetermined amount of a receptor substance having a first part which binds specifically to the analyte, and (ii) a solid phase member having immobilized thereon a ligand which binds specifically to a second part of the receptor, the amount of said ligand on the solid phase member being less than said predetermined amount of the receptor substance.

In still another aspect of the present invention there is provided a test kit for determining an analyte in a sample, comprising (i) a predetermined amount of a receptor substance having a first part which binds specifically to the analyte, only a predetermined fraction of the amount of receptor substance having a second part capable of binding to a specific ligand, and (ii) a solid phase member having said specific ligand immobilized thereon.

In yet another aspect of the present invention there is provided a test kit for determining an analyte in a sample, comprising (i) a first predetermined amount of a receptor substance, and (ii) a solid phase member having immobilized thereon a second predetermined amount of the receptor substance.

While it is preferred to use the method and test kit for quantitative determination of analytes of interest, they may also be used for semi-quantitative and qualitative determinations.

Detailed description of the invention

The essence of the present invention resides in binding all analyte present in a sample to an analyte-specific receptor, isolating a minor fraction of the analyte-receptor complex formed on a solid phase, detecting the amount of isolated analyte-receptor
5 complex, and from this detected amount of analyte on the solid phase determining the total amount of analyte in the sample. According to the invention, this may be accomplished in various ways.

In one embodiment of method of the invention, all analyte is bound by contacting the analyte-containing sample with a solution containing an excess of a first
10 receptor (R1) which in addition to affinity to the analyte has affinity to a ligand (L), whereupon a minor fraction of the analyte-receptor complex is bound to a solid phase having the ligand (L) immobilized thereto. This binding of the minor fraction may be achieved by either (i) using a limited (predetermined) amount of ligand (L) to extract a fraction of the analyte-receptor complex (and unreacted receptor), or (ii) by using a first
15 receptor (R1) only a minor (predetermined) fraction of which is capable of binding to the ligand (L) to extract the desired fraction of the analyte-receptor complex (and unreacted receptor). In the latter case (ii), the amount of immobilized ligand (L) is usually in excess of the amount of the first receptor capable of binding to the ligand (L). The amount of analyte/receptor complex bound to the solid phase is then detected,
20 usually by contacting the solid phase with a detecting agent in the form of a labelled binder for the analyte, such as a labelled second receptor (R2).

In the first case (i) above, the amount of immobilized ligand (L) that can bind to the analyte-specific receptor (R1) is a predetermined fraction of the amount of analyte-specific receptor (R1) contacted with the sample, and therefore the ratio of detected
25 analyte on the solid phase to the total amount of analyte in the sample will correspond to the ratio of immobilized analyte-binding ligand (L) to the total amount of added receptor (R1), thereby permitting the analyte concentration in the sample to be calculated.

In the second case (ii) above, the amount of analyte-specific receptor (R1) that
30 can bind to immobilized ligand (L) is a predetermined fraction of the total amount of receptor (R1), and therefore the ratio of detected analyte on the solid phase to the total amount of analyte in the sample will correspond to the ratio of analyte-specific receptor

(R1) capable of reacting with ligand (L) to the total amount of receptor (R1), thereby permitting the analyte concentration in the sample to be calculated.

The term "receptor" as used herein refers to active analyte-binding receptor, and, where relevant, active ligand-binding receptor, respectively, and is not meant to include such receptor in an inactive or non-binding state. Likewise, the term receptor-binding
5 ligand refers to active receptor-binding ligand and is not meant to include such ligand in an inactive or non-binding state.

The term "amount" as used herein usually means binding capacity. Thus, for example, when it is stated that the amount of analyte-specific receptor is in excess of the
10 amount of analyte, it means that there is more analyte-specific receptor than necessary to bind all analyte. Usually, there is a 1:1 reaction ration between e.g. the analyte and the analyte-specific receptor, or between the analyte-specific receptor and the immobilized receptor-binding ligand. In such a case, the binding capacities of the respective species correspond to their molar amounts. Other reaction ratios are,
15 however, also possible. For example, the immobilized ligand may be capable of binding more than one analyte-specific receptor.

In another embodiment of method of the invention, the sample is contacted with analyte-specific receptor (R1) provided both in solution and, in a minor fraction, immobilized to a solid phase, thereby permitting a minor fraction of analyte present in
20 the sample to be bound to the solid phase. If the ratio of the amount of receptor (R1) in solution to the amount of immobilized receptor (R1) is known, the analyte concentration in the sample may be calculated from the detected amount of analyte bound to the solid phase.

It is readily seen that the above procedure gives the same effect as diluting the
25 sample. In addition to the dilution step being avoided, which, of course, is of advantage to the operator, one obtains a considerable saving in reagents, i.e. both the reagent for capturing the analyte on the solid phase and the detecting agent, the latter often being costly. In this connection, it is also to be noted that in the assay of the invention, the reaction between analyte and receptor takes place in solution where almost all receptors
30 are active rather than at a solid phase surface as in a corresponding conventional assay where only about 10-20% of immobilized receptor will react (Butler, J. E., et al, Molecular Immunology, Vol. 30, No. 13, pp. 1165-1175, 1993).

The required ratio between the total binding capacity of analyte-specific receptor contacted with the sample and (i) the binding capacity of receptor-binding ligand that is immobilized to the solid phase when this is limited, or (ii) the ligand-binding capacity of the analyte-specific receptor when this is limited, is readily determined by the skilled person depending *inter alia* on the particular analyte to be determined and the particular assay format used and may be chosen within wide limits. Usually, this ratio is from about 2:1 to about 1000:1, especially from about 5:1 to about 100:1, preferably more than about 10:1, more preferably more than about 20:1.

The excess of analyte receptor relative to the amount of analyte in the sample is also readily determined by the skilled person for each specific case.

The receptor contacted with the sample is usually of the dual receptor or bireactive binder type having one part that specifically binds to the analyte and another part which specifically binds to the ligand immobilized on the solid phase surface. The analyte binding part may, for example, be an antibody (monoclonal or polyclonal) or an active fragment thereof (including recombinant antibodies and fragments) or nucleic acids whereas the ligand-binding part may be one member of a specific binding pair. Exemplary such specific binding pairs include immunological binding pairs, such as antigen-antibody and hapten-antibody, biotin-avidin or -streptavidin, lectin-sugar, hormone-hormone receptor, and nucleic acid duplex. For example, the solid phase may have streptavidin immobilized thereto, and the receptor for the analyte may be biotinylated. To avoid immunoprecipitation at high analyte concentrations, it may be preferable to use mono-valent receptors.

While the analyte preferably is a molecule present at a high concentration in a sample, the analyte may, of course, be any substance for which there exists a naturally occurring analyte-specific analyte-specific binding member or for which an analyte-specific binding member can be prepared.

Analyte that has been captured by the solid phase is usually detected by reaction with a labelled specific binder for the analyte. Such a labelled binder may be a conjugate comprising a detectable label covalently or non-covalently attached to the specific binding member, "label" referring to any substance which is capable of producing a signal that is detectable by visual or instrumental means, particularly a fluorophore or chromophore.

The sample is usually of biological origin, for example blood (serum, plasma, whole blood), saliva, tear fluid, urine, cerebrospinal fluid, sweat, etc. The invention is, of course, also applicable to other types of samples, such as fermentation solutions, reaction mixtures, etc. Especially, however, the sample is an undiluted serum or whole blood sample.

While the present invention is generally applicable, it may advantageously be used in solid phase assays of the immunochromatographic type. Such assays use a device comprising a plate-shaped flow matrix of bibulous material, usually a membrane strip, such as of cellulose nitrate or glass fiber, in which liquid can be transported laterally (i.e. in the plane of the strip) by capillary forces in the membrane. The membrane usually has a sample application zone, and a detection (or reaction) zone downstream of the sample application zone. In the detection zone, usually a capturing reagent for the analyte is immobilized. To conduct an assay, the application zone is contacted with the liquid sample to be assayed for the analyte of interest. The device is maintained under conditions sufficient to allow capillary action of liquid to transport the analyte of interest, if present in the sample, through the membrane strip to the detection zone where the analyte is captured. The capillary liquid flow is usually insured by an absorbing pad or the like at the downstream end of the strip. A detection reagent, usually labelled, is then added upstream of the detection zone and interacts with captured analyte in the detection zone, and the amount of captured analyte is measured. Often, the detection reagent is pre-deposited in or on the membrane strip, e.g. in the form of diffusively movable particles containing fluorophoric or chromogenic groups, either upstream of the sample application zone or between the sample application zone and the detection zone.

In an immunochromatographic assay according to the invention, the receptor is added to the sample either before applying the sample to the membrane strip, or may be pre-deposited in or on the membrane strip upstream of the detection zone.

A test kit for carrying out the method of the invention may comprise such a membrane having (i) immobilized in or on the membrane a ligand which binds specifically to the receptor, and (ii) dissolvably pre-deposited in or on the membrane a predetermined amount of analyte-specific receptor. The amount of the ligand on the solid phase member is less, and usually considerably less than that required to bind the predetermined amount of the receptor substance.

In another embodiment of test kit, only a fraction of the analyte-specific receptor is capable of binding to the immobilized ligand. Such a kit may comprise (i) immobilized in or on a membrane a ligand which binds specifically to the receptor, and (ii) dissolvably pre-deposited in or on the membrane a predetermined amount of analyte-specific receptor substance, only a predetermined fraction of which is capable of binding to the immobilized ligand.

Still another embodiment of test kit may comprise (i) dissolvably pre-deposited in or on a membrane a first predetermined amount of analyte-specific receptor substance, and (ii) immobilized in or on the membrane a second predetermined amount of the analyte-specific receptor substance.

In an alternative embodiment, the solid phase is a solid phase well, such as a microtiter plate well. Such of test kit may comprise a solid support having one or more wells with the second amount of analyte binding receptor immobilized therein and with the first amount of analyte-binding receptor dissolvably pre-deposited in the well or in close contact with the well.

In the following, the invention will be illustrated in more detail by a specific non-limiting Example.

EXAMPLE 1

Immunoassay for C-reactive protein (CRP) in undiluted serum samples Measuring range 10 – 200 mg/l

Principle

Sample is mixed with biotinylated anti-CRP-fab in excess and the mixture is applied to a test strip having a deficient amount of streptavidin in the reaction zone. After an intermediate wash, anti-CRP fluorophore-conjugate is added and after a wash, conjugate that has bound to the reaction zone is measured. Since only a small part of the biotinylated anti-CRP-fab can bind to the reaction zone the consumption of the fluorophore conjugate is reduced considerably.

Test strips

5 x 48 mm nitrocellulose membranes (Whatman, porosity 8 μ m) on a polyester backing were used. The strips had a sample application zone at one end and a

downstream reaction zone with immobilized streptavidin in an amount capable of binding approximately 6% of biotinylated anti-CRP added in the assay procedure.

Samples

- 5 CRP-containing samples of varying CRP concentration were prepared from a 200 mg/l of recombinant CRP (Fitzgerald) in hCRP depleted serum.

Procedure

- 10 15 µl of biotinylated anti-CRP-fab (monovalent fab-fragment of monoclonal antibody) and 15 µl of CRP-containing serum were mixed and the mixture was applied to the application zone of the membrane strip. The amount of biotinylated anti-CRP-fab was 3 µg per test strip, which is a 2 x molar excess of anti-CRP in relation to the standard 200 mg/l CRP. After an intermediate wash with 15 µl of test buffer (50 mM borate buffer pH 8.0, 3 % BSA, 5 % sucrose, 0.15 M NaCl, 0.005 % CaCl₂, 0.05 %
- 15 NaN₃), 15 µl of detection conjugate solution [3 µg of anti-CRP monoclonal antibody (Fitzgerald) coupled to 0.1 µm TransFluoSpheres-SO₄/CHO (633/720 nm) (Molecular Probes Inc.), the above test buffer] were added , followed by wash with 2 x 15 µl of test buffer. The fluorescence of the strip was then measured. The results are shown in Table 1 below.



Table 1

CRP c nc. (mg/l)	Peak area obtained (V x mm)
0	0.08
0	0.07
10	2.56
10	2.50
30	3.62
30	4.01
100	5.24
100	4.87
200	6.28
200	5.82

EXAMPLE 2 (comparative)

5

Immunoassay for CRP in serum samples diluted 1/20**Measurement range 10 – 200 mg/ml****Principle**

Sample is diluted in test buffer and applied to test strips having an excess of anti-CRP in the reaction zone. Anti-CRP fluorophore-conjugate is then added followed by a wash, whereupon conjugate that has bound to the reaction zone is measured. Sample dilution is necessary to avoid unreasonably large amounts of anti-CRP in the reaction zone as well as in the detection conjugate.

15 **Test strips**

5 x 48 mm nitrocellulose membranes (Whatman, porosity 8 µm) on a polyester backing were used. The strips had a sample application zone at one end and a downstream reaction zone with 2.6 µg immobilized anti-CRP monoclonal antibody (Fitzgerald), which is a 13 x molar excess in relation to a standard 10 mg/ml CRP serum.

20

Samples

CRP-containing samples of varying CRP concentration were prepared from a 200 mg/l of recombinant CRP (Fitzgerald) in hCRP depleted serum.

5 Procedure

15 μ l of CRP-containing serum diluted 1/20 in test buffer (50 mM borate buffer pH 8.0, 3 % BSA, 5 % sucrose, 0.15 M NaCl, 0.005 % CaCl_2 , 0.05 % NaN_3) were applied to the application zone of the membrane strip. Then, 15 μ l of detection conjugate solution [anti-CRP monoclonal antibody (Fitzgerald) coupled to 0.1 μ m
10 TransFluoSpheres-SO₄/CHO (633/720 nm) (Molecular Probes Inc.), the above test buffer] were added, the amount of anti-CRP conjugate being 3 μ g per test strip which was a 15 x molar excess in relation to the highest standard value. The conjugate addition was followed by a wash with 15 μ l of test buffer. The fluorescence of the strip was then measured. The results are shown in Table 2 below.

15

Table 2

CRP conc. (mg/l)	Peak area obtained (V x mm)
0	0.41
0	0.60
10	7.51
10	7.130
20	8.86
20	9.42
40	11.97
40	10.67
80	11.70
80	12.91
200	14.27
200	14.16

The above Examples 1 and 2 thus demonstrate that it is possible to run an assay on undiluted high concentration samples without using huge amounts of reagents when using the methodology of the present invention.

Claims

1. A method of determining an analyte in a sample, especially a high concentration analyte, comprising the steps of:

5

a) contacting the sample with a predetermined amount of a receptor which binds specifically to the analyte to form an analyte/receptor complex, said predetermined amount of receptor being in excess of that required to bind all analyte in the sample,

10

b) isolating on a solid phase a predetermined fraction of the amount of receptor contacted with the analyte, including analyte/receptor complex and unreacted receptor,

c) detecting the amount of analyte/receptor complex in said isolated predetermined fraction, and

15

d) from the detected amount analyte/receptor complex, determining the concentration of analyte in the sample.

20

2. The method according to claim 1, wherein isolating said predetermined fraction of the amount of receptor contacted with the sample on the solid phase comprises providing a solid phase having binding sites for the receptor, and after contacting the sample, or an aliquot thereof, with a liquid phase containing the receptor, binding said predetermined fraction of receptor to the solid phase.

25

3. The method according to claim 2, wherein the whole amount of receptor has reactivity towards said binding sites on the solid phase, and the receptor-binding capacity of the solid phase is less than the solid-phase-binding capacity of receptor contacted with the sample.

30

4. The method according to claim 2, wherein only a predetermined fraction of the amount of receptor contacted with the sample has reactivity towards said binding sites on the solid phase.

5

5. The method according to claim 1, wherein isolating said predetermined fraction of the amount of receptor on the solid phase comprises contacting the sample with a predetermined amount of receptor, a predetermined fraction of which amount is immobilized to said solid phase and the remaining amount of receptor being in a liquid phase.

10

6. The method according to any one of claims 1 to 4, wherein the receptor comprises a first part that binds specifically to the analyte, and a second part that binds to the solid phase.

15

7. The method according to claim 6, wherein the solid phase binding part of the receptor comprises one member of a specific binding pair, and the other member of the binding pair is immobilized to the solid phase.

20

8. The method according to any one the preceding of claims, wherein in step c) the analyte/receptor complex is detected by a labelled detection reagent which binds specifically to the analyte.

25

9. The method according to any one of the preceding claims, wherein the ratio between said isolated fraction of the amount of active analyte-binding receptor and the total amount of active analyte-binding receptor contacted with the sample is in the range of from about 1:2 to about 1:1000, preferably from about 1:5 to about 1:100, particularly no more than about 1:20.

30

10. The method according to any one of the preceding claims, wherein said solid phase binding sites for the receptor are immobilized in a reaction zone of a flow matrix, preferably a lateral flow matrix, such as a membrane strip.

5

11. The method according to any one of the preceding claims, wherein the receptor is an antibody or an immunoactive fragment thereof.

10

12. The method according to any one of the preceding claims, wherein the detection reagent is an antibody or an immunoactive fragment thereof.

15

13. The method according to any one of the preceding claims, wherein the detection reagent is labelled by a fluorophore or a chromophore.

20

14. The method according to any one of the preceding claims, wherein the specific binding pair is biotin-avidin or biotin-streptavidin.

25

15. The method according to any one of the preceding claims, wherein the sample is an undiluted serum sample.

16. The method according to any one of claims 1 to 14, wherein the sample is an undiluted whole blood sample.

30

17. A test kit for determining an analyte in a sample, comprising a predetermined amount of a receptor substance having a first part which binds specifically to the analyte, and a solid phase member having immobilized thereon a ligand which binds specifically to a second part of the receptor, the receptor-binding capacity of said ligand

on the solid phase member being less than the ligand-binding capacity of said predetermined amount of receptor substance.

5 18. The test kit according to claim 17, wherein the ratio between the receptor-binding capacity of ligand immobilized on the solid phase and the ligand-binding capacity of the analyte-specific receptor substance is in the range of from about 1:2 to about 1:1000, preferably from about 1:5 to about 1:100, particularly no more than about 1:20.

10

19. The test kit according to claim 17 or 18, comprising a lateral flow membrane strip having said receptor-binding ligand immobilized in or on a reaction zone of the membrane and having said analyte-binding receptor substance dissolvably pre-
15 deposited in or on the membrane upstream of the reaction zone.

20. A test kit for determining an analyte in a sample, comprising a predetermined amount of a receptor substance having a first part which binds specifically to the
20 analyte, only a predetermined fraction of the amount of receptor substance having a second part capable of binding to a specific ligand, and a solid phase member having said specific ligand immobilized thereon.

25 21. The test kit according to claim 20, wherein the ratio between the amount of ligand-binding analyte-specific receptor and the total amount of analyte-specific receptor is in the range of from about 1:2 to about 1:1000, preferably from about 1:5 to about 1:100, particularly no more than about 1:20.

30

22. The test kit according to claim 20 or 21, comprising a lateral flow membrane strip having said receptor-binding ligand immobilized in or on a reaction zone of the

membrane and having said analyte-binding receptor substance dissolvably pre-deposited in or on the membrane upstream of the reaction zone.

- 5 23. A test kit for determining an analyte in a sample, comprising a first predetermined amount of an analyte-binding receptor substance, and a solid phase member having immobilized thereon a second predetermined amount of said analyte-binding receptor substance.

10

24. The test kit according to claim 23, wherein the ratio between said second amount of analyte-binding receptor substance immobilized to the solid phase, and the sum of said first and second amounts of analyte-binding receptor substance is in the range of from about 1:2 to about 1:1000, preferably from about 1:5 to about 1:100,
15 particularly no more than about 1:20.

25. The test kit according to claim 23 or 24, comprising a lateral flow membrane strip having said second amount of analyte-binding receptor immobilized in or on a
20 reaction zone of the membrane and having said first amount of analyte-binding receptor dissolvably pre-deposited in or on the membrane upstream of the reaction zone.

26. The test kit according to claim 23 or 24, comprising a solid phase well having
25 said second amount of analyte binding receptor immobilized therein and having said first amount of analyte-binding receptor dissolvably pre-deposited in the well or in close contact with the well.

30

ABSTRACT

- 5 The invention relates to a method of determining an analyte in a sample,
especially a high concentration analyte, comprises the steps of:
- a) contacting the sample with a predetermined amount of a receptor which binds
specifically to the analyte to form an analyte/receptor complex, said predetermined
amount of receptor being in excess of that required to bind all analyte in the sample,
 - 10 b) isolating on a solid phase a predetermined fraction of the amount of receptor
contacted with the analyte, including analyte/receptor complex and unreacted receptor,
 - c) detecting the amount of analyte/receptor complex in said isolated predetermined
fraction, and
 - d) from the detected amount analyte/receptor complex, determining the
15 concentration of analyte in the sample.

 The invention also relates to test kits for carrying out the method.